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Eugenol Inhibits K+ Currents in Trigeminal Ganglion Neurons

ABSTRACT

Eugenol, a natural capsaicin congener, is widely used in dentistry. Eugenol inhibits voltage-activated Na^+ and Ca^{2+} channels in a transient receptor potential vanilloid 1 (TRPV1)-independent manner. We hypothesized that eugenol also inhibits voltage-gated K^+ currents, and investigated this in rat trigeminal ganglion neurons and in a heterologous system using whole-cell patch clamping. Eugenol inhibited voltage-gated K^+ currents, and the inhibitory effects of eugenol were observed in both capsaicin-sensitive and capsaicin-insensitive neurons. Pre-treatment with capsazepine, a well-known antagonist of TRPV1, failed to block the inhibitory effects of eugenol on K^+ currents, suggesting no involvement of TRPV1. Eugenol inhibited human Kv1.5 currents stably expressed in *Ltk*$^-$ cells, where TRPV1 is not endogenously expressed. We conclude that eugenol inhibits voltage-gated K^+ currents in a TRPV1-independent manner. The inhibition of voltage-gated K^+ currents is likely to contribute to the irritable action of eugenol. Abbreviations: human Kv1.5 channel, hKv1.5; transient receptor potential vanilloid 1, TRPV1.

KEY WORDS: eugenol, trigeminal ganglion neurons, voltage-gated K^+ currents, Kv1.5.

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INTRODUCTION

Eugenol has been widely used as an analgesic agent in the dental clinic (Markowitz *et al.*, 1992). Recently, we showed that eugenol inhibited voltage-activated Ca^{2+} channels (Lee *et al.*, 2005), as well as voltage-gated Na^+ channel currents and action potentials in dental primary afferent neurons (Park *et al.*, 2006). Voltage-gated channel currents, such as Na^+, sometimes Ca^{2+}, and K^+, play an important role in composing rising and falling phases of action potential. The inhibition of voltage-gated Ca^{2+} and Na^+ currents may contribute to the analgesic effect of eugenol. However, we have also demonstrated that influx of cations through the activation of TRPV1, resulting in depolarization of membrane potential to evoke action potentials, could be one of the major molecular mechanisms by which eugenol produces irritable effects (Yang *et al.*, 2003).

Voltage-gated K^+ channels are a diverse group of membrane proteins that play a fundamental role in determining the electrical properties of excitable cells (Harper and Lawson, 1985). They are essential for information transport and processing by terminating the action potential, repolarizing the neuron, and regulating neurotransmitter release from the pre-synaptic terminal (Rudy, 1988). Capsaicin has been demonstrated, in numerous studies, to inhibit voltage-gated K^+ currents (Liu and Simon, 1996, 2003; Bielefeldt, 2000). Since eugenol, as a natural capsaicin congener, is structurally similar to capsaicin (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), it may modulate voltage-gated K^+ currents in a manner similar to that of capsaicin in sensory neurons (Akins and McCleskey, 1993). In this study, we hypothesized that eugenol can regulate voltage-gated K^+ currents in rat trigeminal ganglion neurons.

MATERIALS & METHODS

All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at the School of Dentistry, Seoul National University.

Preparation of Trigeminal Ganglion Neurons

Trigeminal ganglion neurons from neonatal rats were prepared as previously described (Park *et al.*, 2006). Briefly, trigeminal ganglion neurons were isolated from neonatal (4- to 7-day-old) Sprague–Dawley rats (Samtako BioKorea, Inc., Osan-City, Korea). Dissected trigeminal ganglia were incubated in 3 mL HBSS (Invitrogen, Carlsbad, CA, USA) containing 0.25% trypsin (Invitrogen) at 37°C for 30 min. Neurons were then dissociated by trituration with a series of sterile Pasteur pipettes and, subsequently, were plated onto glass coverslips previously coated with a solution of 0.1 mg/mL poly-L-ornithine (Sigma, St. Louis, MO, USA). Neurons were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C and were used for recording between 6 and 8 hrs after being plated.

Ltk$^-$ Cell Culture

The clonal mouse *Ltk*$^-$ cell line used in this study displayed hKv1.5-specific

mRNA expression after dexamethasone treatment, as evidenced by Northern blot analysis (Tamkun *et al.*, 1991). The transfected cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA), supplemented with 10% horse serum and 0.25 μg/mL G418 (a neomycin analog, Life Technologies) under a 5% CO_2 atmosphere. The cultures were passaged every 3 to 5 days by trypsin dissociation. Before the experiments, the subconfluent cells were incubated with 2 μM dexamethasone for 12 hrs, to induce the expression of hKv1.5 channels, subsequently removed from the dish with a cell scraper. The cell suspension was stored at room temperature (23-25°C) and used within 12 hrs for the experiments.

Electrophysiological Recordings

The patch pipettes were pulled from borosilicate capillaries (Chase Scientific Glass Inc., Rockwood, TN, USA) with resistance of 2~5 MΩ. External solution contained (mM): choline Cl, 70; sucrose, 120; KCl, 5; $MgCl_2 \cdot 6H_2O$, 1; $CoCl_2$, 2; and HEPES, 10. pH was adjusted to 7.4 with choline base. The pipette solution contained (mM): KCl, 120; HEPES, 10; EGTA, 10; $MgCl_2 \cdot 6H_2O$, 2.5; and MgATP, 2. pH was adjusted to 7.4 by KOH. The osmolarity of all solutions used was accurately measured with an Osmometer (Model 3300, Advanced Instruments, Norwood, MA, USA). All of the above chemicals were purchased from Amresco (Solon, OH, USA). Whole-cell currents were recorded with a patch-clamp amplifier (Patch clamp EPC 8, HEKA Elektronik, Lambrecht, Germany), and the signals were filtered at 1 kHz with an eight-pole Bessel filter. By means of an analogue/digital converter (National Instrument Inc., Debrecen, Hungary), the data were stored digitally, and the currents were analyzed with R-clamp 1.23 software [Custom-made patch clamp program with Delphi 5.0 (Boland Inc., Toronto, Canada)]. The current-voltage relationship for the voltage-gated K^+ currents in rat trigeminal ganglion neurons was obtained with a holding potential of -60 mV and voltage steps from -80 mV to +60 mV for 600 ms in 20-mV increments. The effects of various drugs were studied by the use of a holding potential of -80 mV and a single command voltage to 20 mV in 10-second intervals. When measuring the hKv1.5 currents, we used a holding potential of -80 mV and voltage steps from -60 mV to +60 mV for 250 ms in 10-mV increments. In trigeminal ganglion neurons and *Ltk*$^-$ cells, the steady-state currents were obtained at the end of each depolarization pulse. All the experiments were performed at room temperature.

mRNA Preparation and Reverse Transcription/Polymerase Chain-reaction (RT-PCR)

Total RNA was prepared from *Ltk*$^-$ cells and trigeminal ganglion neurons with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized with the Superscript™ Preamplification System (Invitrogen) as previously reported (Fang *et al.*, 2007). PCR reaction was performed with 2 μL of the resulting cDNA with Taq DNA polymerase (Invitrogen), and primers for PCR were specifically designed for TRPV1 based on Genebank mouse cDNA sequences. The primers (forward/reverse) used for the amplification of TRPV1 and β-actin were AGCGAGTTCAAAGACCCAGA/TTCTCCACCAAGAGGGTCAC and AAGAGACAACATTGGCATGG/TGAGGGACTTCCTGTAACCA, respectively. PCR reactions with both cDNA from neonatal rat trigeminal ganglion neurons and water were run in parallel as positive and negative controls, respectively.

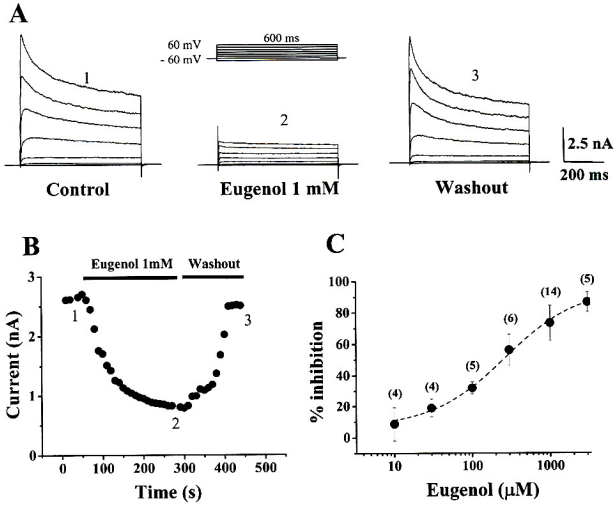

Figure 1. Eugenol inhibited voltage-gated K^+ currents in rat trigeminal ganglion neurons. **(A)** Using pulses from -80 mV to 60 mV for 600 ms with a holding potential of -60 mV, we obtained the representative traces under control, 1 mM eugenol, and wash-out conditions, measured at the points indicated in (B). **(B)** For time-course recording of voltage-gated K^+ currents, we used a depolarizing potential of 20 mV from a holding of -80 mV in 10-second intervals. When trigeminal ganglion neurons were exposed to eugenol (1 mM), the voltage-gated K^+ current was readily inhibited in a reversible manner. **(C)** The dose-response curve of the inhibition of voltage-gated K^+ currents by eugenol with the IC_{50} of 376 ± 90 μM. The number in parentheses represents the number of cells studied.

Drugs

Eugenol and capsazepine, purchased from Sigma, were dissolved in dimethylsulfoxide (DMSO) to make a stock solution, and kept at -20°C. The drugs were diluted to their final concentration in the extracellular solution, and then applied by gravity through a bath perfusion system. Most neurons were exposed to only a single dose of eugenol, and the results were averaged across neurons. The sensitivity of eugenol-responsive cells to capsaicin was determined based on the inhibition of voltage-gated K^+ currents by 1 μM capsaicin, either before (n = 41) or after (n = 20) the exposure of cells to eugenol. The perfusion rate of the bath solution was continuous (1 mL/min) during the experiment.

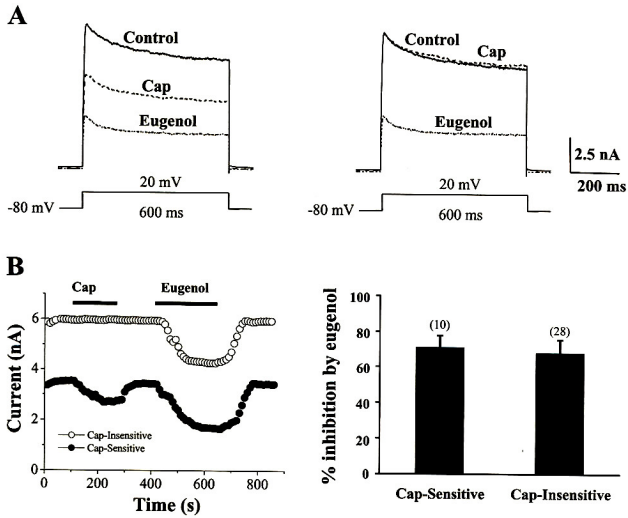

Figure 2. Eugenol inhibited voltage-gated K^+ currents in both capsaicin-sensitive and capsaicin-insensitive rat trigeminal ganglion neurons. **(A)** Representative current traces of voltage-gated K^+ currents under control, eugenol (1 mM), and capsaicin (1 μM), respectively, in 2 trigeminal ganglion neurons. Eugenol (1 mM) inhibited voltage-gated K^+ currents in both capsaicin-sensitive (left) and capsaicin-insensitive (right) trigeminal ganglion neurons. **(B)** Representative time-courses of the inhibition of voltage-gated K^+ currents by eugenol in capsaicin-sensitive (black circle) and capsaicin-insensitive (white circle) trigeminal ganglion neurons (left). The summary of the inhibition of voltage-gated K^+ currents in trigeminal ganglion neurons (right) indicated that eugenol (1 mM)-induced inhibition in capsaicin-insensitive neurons was similar to that obtained in capsaicin-sensitive neurons (mean ± SEM, $p > 0.05$). The number in parentheses represents the number of cells studied.

Statistical Analyses

Data are expressed as mean ± SEM. We used ANOVA and Student's *t* test to determine the differences, using the software Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA). Differences were considered to be significant when p was less than 0.05.

RESULTS

Eugenol Inhibited Voltage-gated K^+ Currents in Rat Trigeminal Ganglion Neurons

In this study, we used acutely isolated trigeminal ganglion neurons ranging from 20 μm to 45 μm in diameter. Eugenol (1 mM) inhibited voltage-gated K^+ currents in a reversible manner (Figs. 1A, 1B). When trigeminal ganglion neurons were exposed to eugenol, the voltage-gated K^+ currents were readily inhibited in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) was 376 ± 90 μM (Fig. 1C).

Eugenol Inhibited Voltage-gated K^+ Currents in Both Capsaicin-sensitive and -insensitive Rat Trigeminal Ganglion Neurons

We then tested if the inhibitory effect of eugenol on voltage-gated K^+ currents is specific only to capsaicin-sensitive trigeminal ganglion neurons. We observed that eugenol inhibited voltage-gated K^+ currents in both capsaicin-sensitive and -insensitive neurons (Figs. 2A, 2B). The magnitude of the inhibition of voltage-gated K^+ currents by 1 mM eugenol was similar between capsaicin-insensitive (68 ± 8%, n = 28) and capsaicin-sensitive neurons (71 ± 7%, n = 10) (Fig. 2B, right).

Inhibition of Voltage-gated K^+ Currents by Eugenol Does Not Require TRPV1 Activation

To determine whether the inhibitory effect of eugenol on voltage-gated K^+ currents was mediated by TRPV1, we examined the effects of eugenol in the presence of 10 μM capsazepine (Chaudhary *et al.*, 2001). When we applied 10 μM capsazepine, a competitive TRPV1 antagonist, for 5 min, it did not produce inhibitory effects on voltage-gated K^+ currents (n = 8) (Fig. 3A), although it has been previously reported that capsazepine may inhibit Ca^{2+} currents (Docherty *et al.*, 1997). The application of 10 μM capsazepine together with 1 mM eugenol inhibited voltage-gated K^+ currents (73 ± 8%, n = 15), which were not significantly different from the inhibition by eugenol alone (69 ± 8%, n = 35) (Fig. 3A).

To determine further whether the inhibitory effect of eugenol on voltage-gated K^+ currents was mediated by TRPV1, we tested the inhibitory effects of eugenol on voltage-gated K^+ currents in Ltk^- cells that stably express hKv1.5 channels. RT-PCR analysis indicated that TRPV1 was not endogenously expressed in Ltk^- cells, whereas it was expressed in trigeminal ganglion neurons (Fig. 3B, insert). In Ltk^- cells, 1 mM eugenol still produced an inhibitory effect on hKv1.5 currents, although TRPV1 was not endogenously expressed (Fig. 3B, left). The extent of inhibition by 1 mM eugenol was greater in trigeminal ganglion neurons (69 ± 8%, n = 35) than in Ltk^- cells (43 ± 7%, n = 6) (Fig. 3B, right).

DISCUSSION

We have recently demonstrated that eugenol inhibits voltage-gated Ca^{2+} currents in dental primary afferent neurons in a TRPV1-independent manner (Lee *et al.*, 2005). We also reported that eugenol inhibited voltage-gated Na^+ currents, thereby blocking the generation of action potentials in dental primary afferent neurons, and these effects were observed in both capsaicin-sensitive and -insensitive neurons (Park *et al.*, 2006). It was concluded that the inhibition of voltage-gated Ca^{2+} and Na^+ currents contributes to the analgesic effect of

eugenol. In this study, we investigated whether eugenol can also regulate voltage-gated K^+ currents in rat trigeminal ganglion neurons. Because of the difficulty in accessing peripheral nerve endings by electro-physiological methods or other functional approaches, studies on peripheral sensory functions of ion channels have been mainly performed on the somas of primary afferent neurons, such as trigeminal ganglion neurons (Liu and Simon, 1996, 2003; Komai and McDowell, 2001). It has been generally accepted that if a certain protein is expressed on a soma of a primary afferent neuron, the peripheral nerve endings of the primary afferent fiber also express the same protein (Nakatsuka and Gu, 2006). We found that eugenol elicited inhibitory effects on voltage-gated K^+ currents in all rat trigeminal ganglion neurons tested, which include both capsaicin-sensitive and -insensitive neurons.

These findings imply that, although eugenol and capsaicin share a vanillyl-like moiety in their chemical structure (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), the mechanisms underlying the inhibitory effects of eugenol and capsaicin on voltage-gated K^+ currents may differ. Capsaicin reduces fast-inactivating A-type currents (I_A), one type of voltage-gated K^+ current, through TRPV1 in rat trigeminal ganglion neurons (Liu and Simon, 2003), indicating the involvement of TRPV1 in the inhibitory effects of capsaicin on I_A currents. However, we found that eugenol inhibited voltage-gated K^+ currents in both capsaicin-sensitive and –insensitive neurons. Moreover, capsazepine, a competitive TRPV1 antagonist, failed to block the inhibitory effects of eugenol on voltage-gated K^+ currents, showing that the inhibitory effects on voltage-gated K^+ currents produced by eugenol were not mediated by TRPV1. The TRPV1-independence of eugenol action on voltage-gated K^+ currents was further confirmed with a heterologous system. In Ltk^- cells, which were transfected by human Kv1.5 and did not contain endogenous TRPV1, eugenol produced inhibitory effects on Kv1.5 currents, one of the major subtypes of voltage-gated K^+ currents in sensory neurons (Andrews and Kunze, 2001; Duzhyy *et al.*, 2004). This clearly demonstrates that TRPV1 activation is not involved in the eugenol-induced inhibitory effects on voltage-gated K^+ currents. Given that the inhibitory effects of eugenol on K^+ currents were greater in trigeminal ganglion neurons than in the Ltk^- cells in our study, it is highly likely that eugenol might exert inhibitory effects on other subtypes of K^+ channels, in addition to Kv1.5 channels, which exist in rat trigeminal ganglion neurons. Indeed, Liu and Simon demonstrated the expression of diverse subtypes of voltage-gated K^+ channels, including Kv1.5, in trigeminal ganglion neurons (Liu and Simon, 2003).

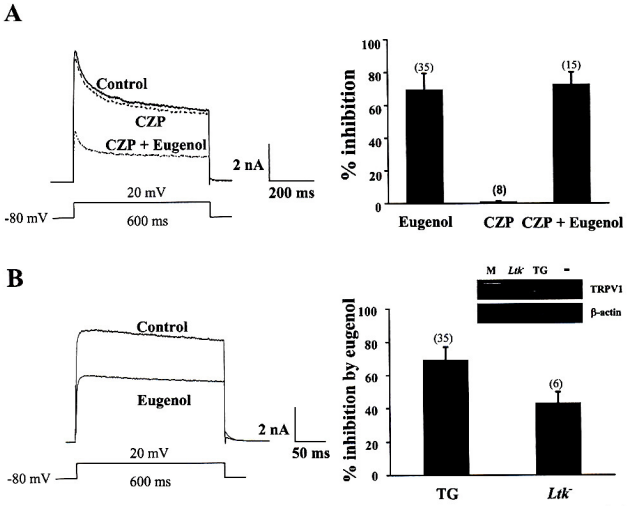

Figure 3. Inhibition of voltage-gated K^+ currents by eugenol does not require TRPV1 activation. **(A)** Capsazepine (CZP) did not block the inhibition of voltage-gated K^+ currents by eugenol in rat trigeminal ganglion (TG) neurons. The representative traces of voltage-gated K^+ currents under control, CZP (10 μM), and the combined application of eugenol and CZP (left). The inhibition of voltage-gated K^+ currents by the combined application of eugenol and CZP was not significantly different from that of eugenol only (mean ± SEM, $p > 0.05$) (right), indicating that eugenol-induced voltage-gated K^+ current inhibition was TRPV1-independent. The number in parentheses represents the number of cells studied. **(B)** Representative current traces under control and 1 mM eugenol in Ltk^- cells (left). Eugenol inhibited hKv1.5 currents in Ltk^- cells. The summary of K^+ current inhibition in trigeminal ganglion neurons and Ltk^- cells (right). The K^+ current inhibition by eugenol in trigeminal ganglion neurons was significantly greater than that in Ltk^- cells (mean ± SEM, $p < 0.05$). (Insert) RT-PCR analysis shows no endogenous expression of TRPV1 in Ltk^- cells. TRPV1 is clearly expressed in trigeminal ganglion neurons. The expected sizes of PCR products were 233 bp (TRPV1) and 293 bp (β-actin). No PCR products were detected with H_2O (lane –).

According to our previous studies, we found that eugenol has both excitatory and inhibitory effects. The inhibitory effects of eugenol on voltage-gated Ca^{2+} currents (Lee *et al.*, 2005) and voltage-gated Na^+ currents in dental primary sensory neurons (Park *et al.*, 2006) may contribute to its analgesic effects. We have also demonstrated an excitatory effect of eugenol that evokes influx of cations thorough TRPV1, which may be one of the molecular mechanisms by which eugenol produces irritable actions (Yang *et al.*, 2003). In this study, we suggest another mechanism underlying the irritable action of eugenol by showing that eugenol inhibits voltage-gated K^+ currents. The inhibition of K^+ currents may decrease the efficiency of terminating action potential and lead to the elongation of action potential duration and the relative refractory period. Indeed, we found the prolongation of action potential duration by eugenol in a subpopulation (~ 13%) of

trigeminal ganglion neurons tested (n = 54, data not shown).

As to its inhibitory effects on the voltage-gated Na^+, Ca^{2+}, and K^+ currents in trigeminal ganglion neurons, the ranges of eugenol concentrations and extent of inhibitory percentage by eugenol showed that voltage-gated K^+ currents were less sensitive to eugenol than were voltage-gated Na^+ currents, and both currents were much more sensitive than voltage-gated Ca^{2+} currents (Lee *et al.*, 2005; Park *et al.*, 2006).

In summary, we have demonstrated that eugenol produces inhibitory effects on voltage-gated K^+ currents in rat trigeminal ganglion neurons, and that TRPV1 activation is not a prerequisite for the inhibitory effects of eugenol. The inhibition of voltage-gated K^+ currents is likely to contribute to the irritable action of eugenol.

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